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Stimulation of Inositol 1,4,5-Trisphosphate (IP₃) Receptor Subtypes by Adenophostin A and Its Analogues

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃R) are intracellular Ca²⁺ channels. Most animal cells express mixtures of the three IP₃R subtypes encoded by vertebrate genomes. Adenophostin A (AdA) is the most potent naturally occurring agonist of IP₃R and it shares with IP₃ the essential features of all IP₃R agonists, namely structures equivalent to the 4,5-bisphosphate and 6-hydroxyl of IP₃. The two essential phosphate groups contribute to closure of the clam-like IP₃-binding core (IBC), and thereby IP₃R activation, by binding to each of its sides (the α - and β -domains). Regulation of the three subtypes of IP₃R by AdA and its analogues has not been examined in cells expressing defined homogenous populations of IP₃R. We measured Ca²⁺ release evoked by synthetic adenophostin A (AdA) and its analogues in permeabilized DT40 cells devoid of native IP₃R and stably expressing single subtypes of mammalian IP₃R. The determinants of high-affinity binding of AdA and its analogues were indistinguishable for each IP₃R subtype. The results are consistent with a cation- π interaction between the adenine of AdA and a conserved arginine within the IBC α -domain contributing to closure of the IBC. The two complementary contacts between AdA and the α -domain (cation- π interaction and 3''-phosphate) allow activation of IP₃R by an analogue of AdA (3''-dephospho-AdA) that lacks a phosphate group equivalent to the essential 5-phosphate of IP₃. These data provide the first structure-activity analyses of key AdA analogues using homogenous populations of all mammalian IP₃R subtypes. They demonstrate that differences in the Ca²⁺ signals evoked by AdA analogues are unlikely to be due to selective regulation of IP₃R subtypes.

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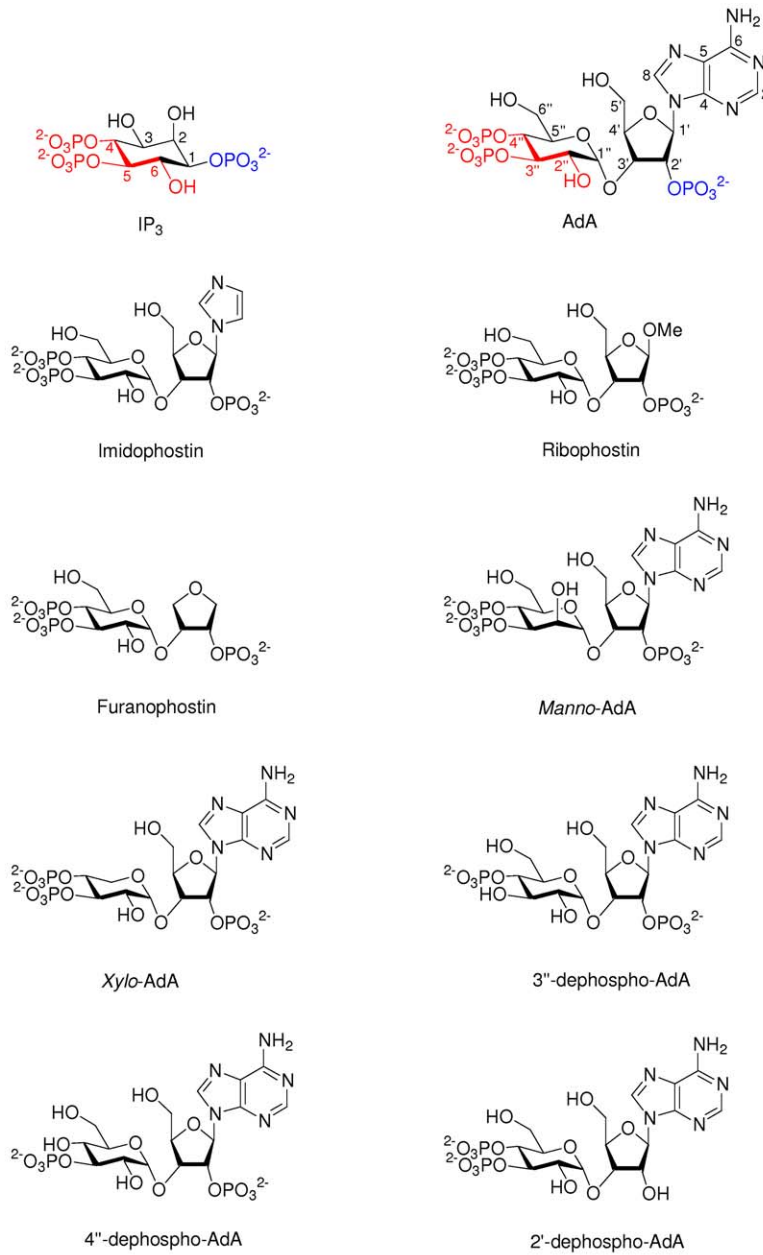
Introduction

Inositol 1,4,5-trisphosphate receptors (IP₃R) are intracellular Ca²⁺ channels that are expressed in almost all animal cells. They allow release of Ca²⁺ from intracellular stores in response to the many stimuli that activate phospholipase C [1,2]. The genomes of vertebrates encode three closely related IP₃R subtypes (IP₃R1-3), and most cells from vertebrates express functional IP₃R that are homo- or hetero-tetrameric assemblies of these IP₃R subtypes and their splice variants [3]. The physiological significance of this IP₃R diversity is poorly understood, and nor are there ligands that usefully discriminate between IP₃R subtypes. It is, however, clear that activation of IP₃R is initiated by binding of IP₃ to the conserved IP₃-binding core (IBC, residues 224-604 of IP₃R1) of each IP₃R subunit [4]. Mixed populations of IP₃R in native cells make it difficult to define unambiguously the functional properties of each IP₃R subtype. Stable heterologous expression of mammalian IP₃R in the only vertebrate cell line engineered to lack all endogenous IP₃R (DT40 KO cells) [5] provides an effective means of addressing this difficulty [6]. We previously used DT40 cells expressing homogeneous populations of each mammalian IP₃R subtype to define structure-activity relationships for key endogenous and synthetic inositol phosphates [7]. Here, we extend the approach to examine the interactions of each IP₃R subtype with

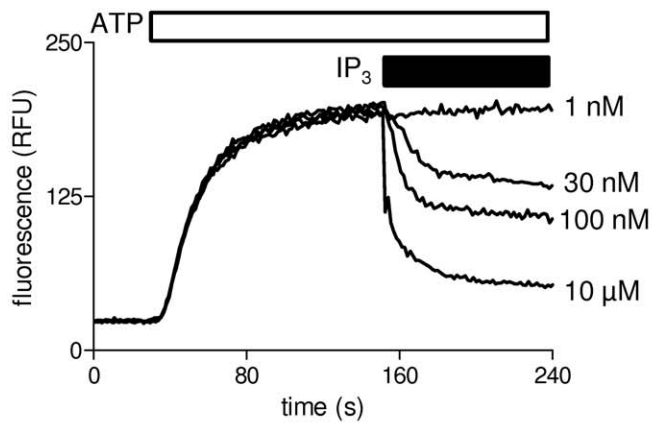
adenophostin A (**1**, AdA) and its most important analogues [8] (Figure 1A).

AdA, originally isolated from *Penicillium brevicompactum* [9,10] and later synthesized [11], is a potent agonist of IP₃R. It is also resistant to degradation by the enzymes that degrade IP₃ via phosphorylation or dephosphorylation [10]. Although AdA is based on a glucose ring, rather than the inositol ring of IP₃, its structure retains the key functional groups of IP₃ that are known to be essential for IP₃ activity at IP₃R [12] (Figure 1A). Considerable evidence supports the original suggestion [10] that the essential 4,5-bisphosphate and 6-hydroxyl of IP₃ are effectively mimicked by the 4'',3''-bisphosphate and 2''-hydroxyl of AdA (red highlights in Figure 1A). The interactions that allow AdA to bind to IP₃R with about 10-fold greater affinity than IP₃ have been more difficult to resolve. One view was that the 2'-phosphate of AdA is equivalent to the 1-phosphate of IP₃ and, like the latter [13] (blue in Figure 1A), contributes to high-affinity binding to the IBC. The suggestion was that the 2'-phosphate of AdA forms a stronger interaction with the IBC than does the 1-phosphate of IP₃. Our recent analyses have challenged this idea and instead suggest that a cation- π interaction between the adenine ring of AdA and a guanidinium side chain of an arginine residue within the α -domain of the IBC (R504 in IP₃R1) may be a more important determinant of the increased affinity of AdA for IP₃R [12].

A



B



C

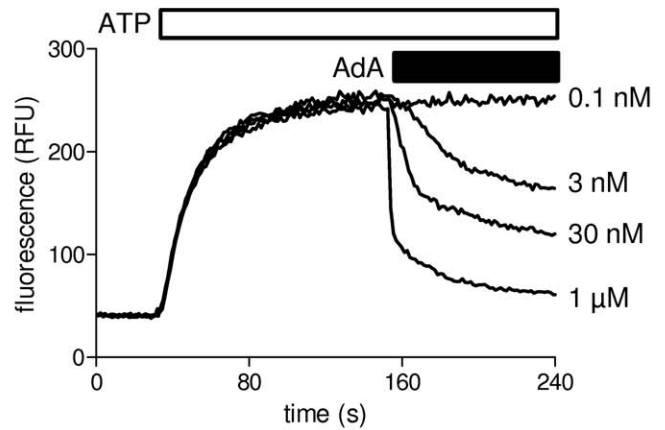


Figure 1. Structures of the analogues of AdA used. (A) Key moieties within IP₃ and AdA are highlighted in matching colours to indicate their proposed structural equivalence. (B and C). The Ca²⁺ contents of the intracellular stores of populations of permeabilized DT40-IP₃R1 cells are shown after addition of ATP to allow active Ca²⁺ uptake, and then addition of the indicated concentrations of IP₃ or AdA with thapsigargin (1 μM) to inhibit further Ca²⁺ uptake. The traces, which are typical of those from all subsequent analyses, show the average response from 2 wells on a single plate. The results demonstrate that both IP₃ and AdA evoke quantal Ca²⁺ release.
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The high-affinity and metabolic stability of AdA have generated considerable interest in both the synthesis of AdA analogues and their application to analyses of IP₃R activation and associated changes in cytosolic Ca²⁺ signalling [12]. There has, however, been no systematic analysis of the activities of AdA or its analogues with defined populations of homogenous IP₃R subtypes. The need for such analyses is particularly important in attempting to explain results in which Ca²⁺ signals evoked by IP₃ differ from those evoked by AdA [14,15,16,17,18,19,20,21], or where different analogues of AdA evoke different cellular responses [reviewed in 12,22]. Here we use DT40 cells in which all endogenous IP₃R have been genetically inactivated [5] to stably express homogenous populations of mammalian IP₃R subtypes and thereby define structure-activity relationships for AdA and its key analogues for each IP₃R subtype.

Materials and Methods

Materials

Sources of most reagents were provided in a previous publication [7]. The structures of the ligands used and their abbreviations are shown in Figure 1A. IP₃ was from Alexis Biochemicals (Nottingham, UK). AdA [23], imidophostin [24], ribophostin [25], furanophostin [26], *manno*-AdA and *xylo*-AdA [27], 3''-dephospho AdA and 4''-dephospho AdA [28], and 2'-dephospho AdA were synthesized, purified and characterized as previously described.

Measurement Ca²⁺ Release by IP₃ Receptors

From quantitative analyses of western blots using antisera that selectively recognise each IP₃R subtype or react equally with all three subtypes, we established that in the DT40 cells used, levels of IP₃R expression (relative to IP₃R3) were IP₃R1 (71±8%, n=3), IP₃R2 (48±5%) and IP₃R3 (100%) [7]. It is impracticable to achieve identical levels of IP₃R expression for each cell line, and differences (albeit modest in our cell lines) may affect both the size of the IP₃-sensitive Ca²⁺ pool and its sensitivity to IP₃ [29]. The different levels of IP₃R expression do not compromise the analyses reported here, which are entirely concerned with relative potencies of AdA analogues for each IP₃R subtype (see below).

A comprehensive description of the methods used to measure free [Ca²⁺] within the endoplasmic reticulum of permeabilized DT40 cells was provided in preceding publications [7,30]. Briefly, the endoplasmic reticulum of DT40 cells stably expressing each of the three mammalian IP₃R subtypes was loaded with a low-affinity Ca²⁺ indicator (Mag fluo-4) [30]. After permeabilization of the plasma membrane with saponin (10 μg/mL, ~4 min, 37°C), the permeabilized cells in cytosol-like medium (CLM) were distributed into 96-well plates at 20°C. Addition of MgATP (1.5 mM) then allowed active Ca²⁺ accumulation, which was monitored at intervals of ~1 s using a FlexStation 3 fluorescence plate-reader (MDS Analytical Devices). CLM had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 20 mM Pipes, pH 7, free [Ca²⁺] ~220 nM (after addition of MgATP), and carbonyl cyanide 4-trifluoromethoxy-phenyl hydrazone (FCCP, 10 μM) to inhibit mitochondrial Ca²⁺ uptake. After 150 s, when the stores had loaded to steady-state with Ca²⁺, IP₃, AdA or its analogues

was added with thapsigargin (1 μM) to prevent further Ca²⁺ uptake, and after a further 30 s, the response was recorded. Agonist-evoked Ca²⁺ release was expressed as a fraction of that released by ionomycin (1 μM) [30]. All experiments were performed at 20°C.

Statistical Analysis

Concentration-effect relationships were fitted to Hill equations using GraphPad Prism (version 5.0) from which Hill coefficients (h), the fraction of the intracellular Ca²⁺ stores released by maximally effective concentrations of agonist, and pEC₅₀ values (-log EC₅₀) were calculated. For convenience some results are presented as EC₅₀ values, but all statistical comparisons use pEC₅₀ values. Within each experiment, the pEC₅₀ for AdA was determined to allow paired comparisons with values obtained for each AdA analogue. These are reported as ΔpEC₅₀, where:

$$\Delta pEC_{50} = pEC_{50}^{AdA} - pEC_{50}^{analogue}$$

We note that Table 1 reports pooled results from experiments collected over a considerable period, whereas ΔpEC₅₀ values, like those shown in Table 2, compare only paired values. The latter provide the most robust means of comparing agonist potencies. Results are expressed as means ± SEM from n independent experiments, with each experiment performed in triplicate.

Statistical comparisons used Student's t-test or ANOVA followed by Bonferroni's *post hoc* test, as appropriate, with *P*<0.05 considered significant. Because not all comparisons of the relative potencies of AdA and IP₃ were paired, the SEM of this ΔpEC₅₀ value was calculated from:

$$SEM = s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where, *s_p* is the estimate of the population variance:

$$s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

where, *s₁* and *s₂* are the sample standard deviations, and *n₁* and *n₂* are the sample sizes [31].

Results

Quantal Ca²⁺ Release Evoked by AdA and IP₃

The kinetics of IP₃-evoked Ca²⁺ release from intracellular stores are unexpectedly complex. It is widely observed that under conditions where Ca²⁺ uptake into the endoplasmic reticulum (ER) is inhibited, submaximally effective concentrations of IP₃ rapidly release only a fraction of the IP₃-sensitive Ca²⁺ stores [32]. Thereafter, there is either no, or a massively reduced, effect of IP₃ on the rate of Ca²⁺ release. The mechanisms underlying this pattern of response, known as quantal Ca²⁺ release [33], remain

Table 1. Effects of AdA analogues on Ca²⁺ release by subtypes of IP₃ receptor.

	IP ₃ R1					IP ₃ R2					IP ₃ R3				
	EC ₅₀	pEC ₅₀	h	Ca ²⁺ release	n	EC ₅₀	pEC ₅₀	h	Ca ²⁺ release	n	EC ₅₀	pEC ₅₀	h	Ca ²⁺ release	n
(1,4,5)IP ₃	87	7.06±0.05	0.99±0.05	75±1	31	145	6.84±0.06	1.29±0.09	61±2	34	417	6.38±0.05	1.26±0.07	64±2	30
AdA	8.3	8.08±0.09	1.17±0.09	72±3	10	18.2	7.74±0.06	1.79±0.21	56±2	13	33	7.48±0.09	1.13±0.07	61±2	14
Imidophostin	37	7.43±0.28	1.17±0.21	78±5	3	68	7.17±0.14	1.84±0.50	59±3	3	166	6.78±0.16	1.73±0.39	67±7	3
Ribophostin	40	7.40±0.29	1.34±0.16	77±4	3	102	6.99±0.11	1.60±0.50	61±2	3	295	6.53±0.21	1.42±0.08	68±4	3
Furanophostin	51	7.29±0.25	0.90±0.10	79±6	3	76	7.12±0.01	1.73±0.20	60±3	3	457	6.34±0.18	1.27±0.21	71±3	3
Manno-AdA	34	7.47±0.19	1.33±0.30	75±7	3	69	7.16±0.07	1.33±0.22	57±3	3	245	6.61±0.23	1.23±0.15	69±4	3
Xylo-AdA	5.9	8.23±0.17	1.27±0.27	73±7	3	7.9	8.10±0.10	1.52±0.40	52±6	3	29	7.54±0.12	1.58±0.29	64±9	3
2'-dephospho-AdA	275	6.56±0.13	1.31±0.15	66±7	3	575	6.24±0.10	0.85±0.07	63±2	3	692	6.16±0.03	1.5±0.22	55±7	4
3"-dephospho-AdA ^c	ND	ND	ND	15±6 ^b	7	ND	ND	ND	6±2 ^b	6	ND	ND	ND	7±7 ^b	5
4"-dephospho-AdA	Inactive ^a	Inactive ^a	Inactive ^a	ND	6	Inactive ^a	Inactive ^a	Inactive ^a	ND	6	Inactive ^a	Inactive ^a	Inactive ^a	ND	5

The EC₅₀ (nM), pEC₅₀ (/M), Hill coefficient (h) and fraction (%) of the intracellular Ca²⁺ stores released by a maximally effective concentration of each analogue are shown for each IP₃R subtype. All results (except EC₅₀) are shown as means ± SEM from n independent experiments.

^aInactive at 300 μM.

^bCa²⁺ release evoked by 300 μM 3"-dephospho AdA.

^cRefer to Table 2 for relative potencies of 3"-dephospho AdA. ND, not determined.

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Table 2. Relative potencies of AdA analogues at different IP₃ receptor subtypes.

	IP ₃ R1	IP ₃ R2	IP ₃ R3
IP ₃	1.02±0.02	0.9±0.30	1.1±0.30
Imidophostin	0.78±0.15	0.78±0.08	0.81±0.04
Ribophostin	0.82±0.18	0.96±0.20	1.06±0.07
Furanophostin	0.92±0.13	0.83±0.14	1.25±0.05
Manno-AdA	0.74±0.08	0.79±0.18	0.98±0.08
Xylo-AdA	-0.01±0.07	-0.3±0.27	0.05±0.08
2'-dephospho-AdA	1.24±0.33	1.60±0.18	1.68±0.16
3"-dephospho-AdA ^a	4.03±0.09	4.47±0.30	4.13±0.14

From paired comparisons with AdA, the potency (ΔpEC_{50}) of the analogues relative to AdA is shown for each IP₃R subtype. Results are means \pm SEM, with n provided in Table 1. ND, not determined. ^aBecause the very low affinity of 3"-dephospho AdA for IP₃R made it impracticable to stimulate cells with a maximally effective concentration, ' ΔpEC_{50} ' for 3"-dephospho AdA was estimated by comparing concentrations of it and AdA that evoked the same sub-maximal Ca²⁺ release.

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unclear. It may require desensitization of IP₃R as the Ca²⁺ content of the ER declines [34] or heterogeneity among IP₃-sensitive Ca²⁺ stores [35]. The results shown in Figures 1B and C confirm that the Ca²⁺ release evoked by submaximal concentrations of either IP₃ or AdA from permeabilized DT40-IP₃R1 cells is quantal. These observations provide the justification for all subsequent experiments in which the concentration-dependent effects of IP₃ or AdA were measured 30 s after their addition (see Methods).

AdA is a Potent Agonist of All Three IP₃ Receptor Subtypes

The results shown in Figure 2 and Tables 1 and 2 demonstrate that AdA is ~10-times more potent than IP₃ at each IP₃R subtype, and for each subtype, maximally effective concentrations of IP₃ and AdA release the same fraction of the intracellular Ca²⁺ stores. This is consistent with many analyses of IP₃ and AdA in a variety of cell types using both functional and binding assays, in which AdA behaves as a full agonist with ~10-fold greater affinity than IP₃ [reviewed in 8]. Our results do, however, provide the first direct demonstration that AdA interacts similarly with all three IP₃R subtypes. Subsequent experiments examine the interactions between key analogues of IP₃ and AdA with each IP₃R subtype.

Trimming the Adenosine Moiety of AdA Reduces its Potency at All IP₃ Receptor Subtypes

Systematic trimming of the adenosine moiety of AdA successively produces imidophostin (which lacks the pyrimidine ring of AdA), ribophostin (in which a methoxy group replaces the adenine moiety of AdA) and furanophostin (in which only the furanoid ring remains) (Figure 1A). Maximally effective concentrations of each of these analogues released the same fraction of the intracellular Ca²⁺ stores as AdA in cells expressing each of the three IP₃R subtypes, and each analogue was ~5-10-fold less potent than AdA (Figure 3, Tables 1 and 2). These results are consistent with previous analyses of IP₃R in hepatocytes, which express predominantly IP₃R2 [24,36], with analyses of binding of ribophostin and furanophostin to an N-terminal fragment of IP₃R1 [12], and with evidence from other analogues that trimming the adenosine

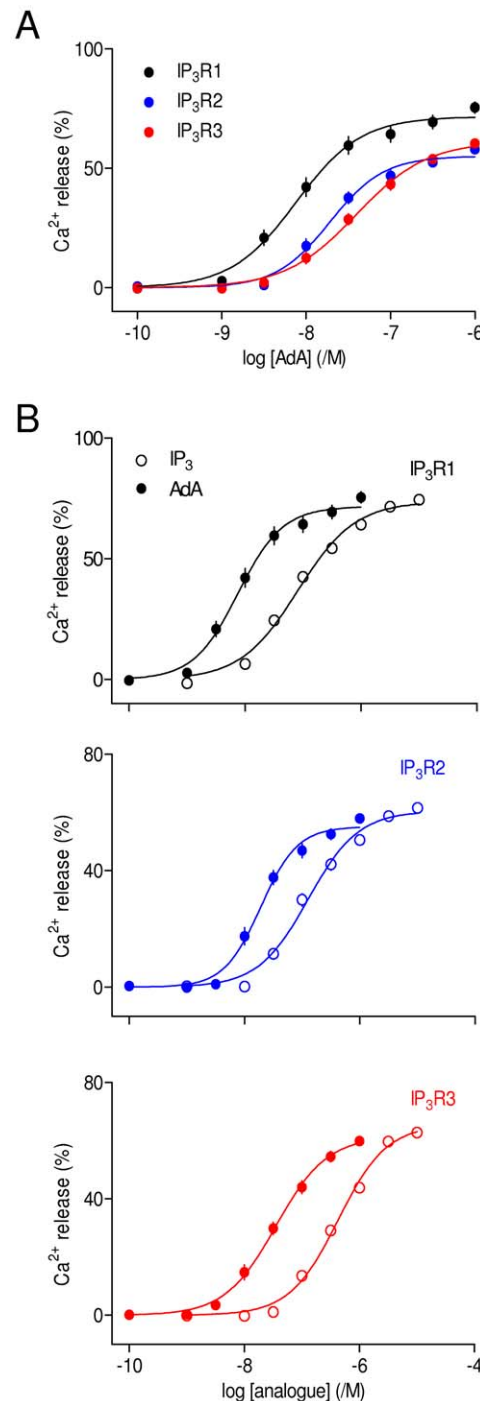


Figure 2. AdA is a potent agonist of all three IP₃ receptor subtypes. (A) Concentration-dependent effects of AdA on Ca²⁺ release from the intracellular stores of cells expressing IP₃R1, IP₃R2 or IP₃R3. All results are expressed as percentages of the Ca²⁺ release evoked by ionomycin. The same colour codes are used in all subsequent figures. (B) Comparison, for each IP₃R subtype, of the Ca²⁺ release evoked by IP₃ and AdA. Results are means \pm SEM from the number of independent experiments given in Table 1. Here, and in many subsequent figures, some error bars are smaller than the symbols.

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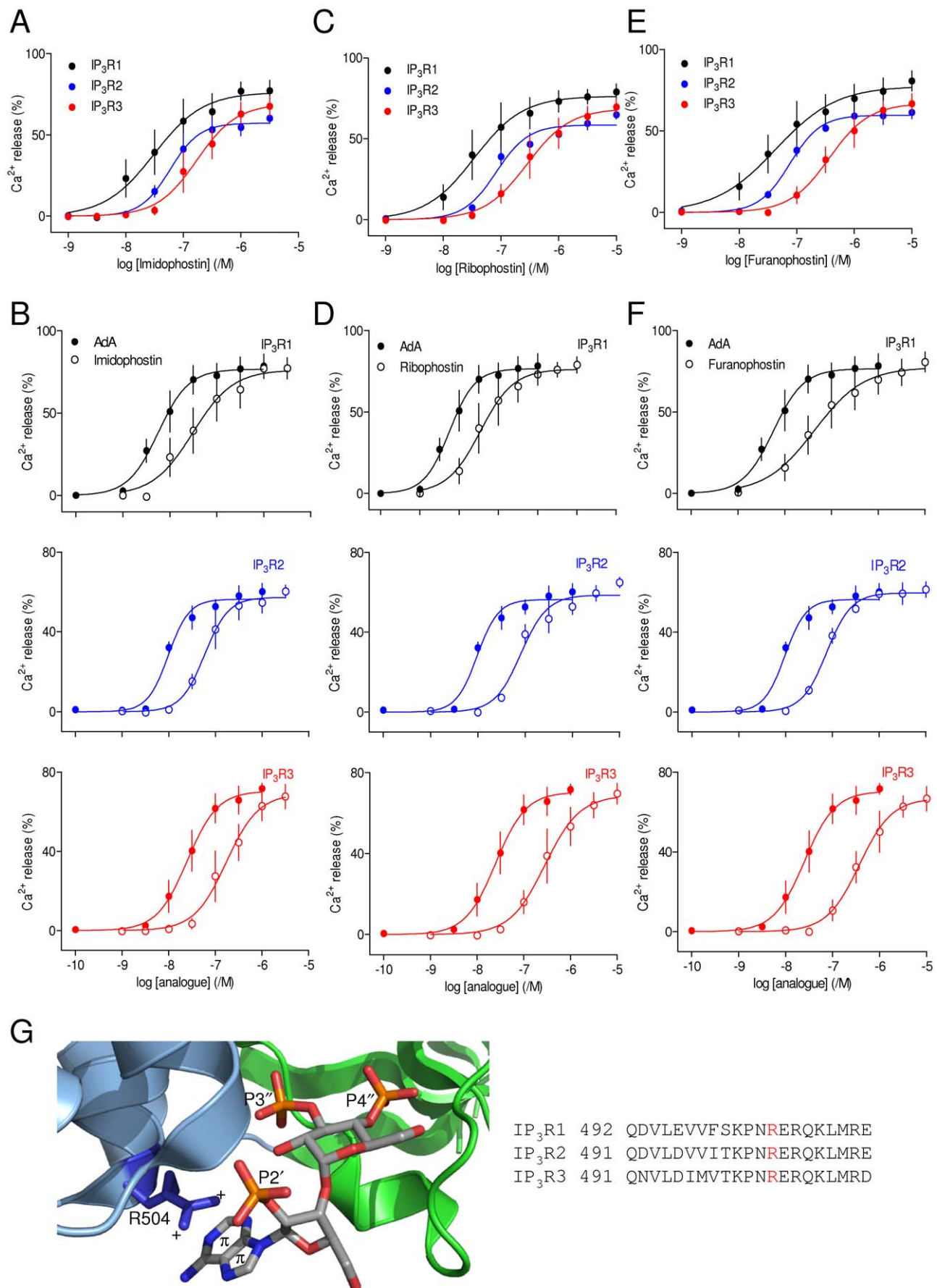


Figure 3. Trimming the adenosine moiety of AdA reduces potency. (A–F) Effects of imidophostin (A), ribophostin (C) and furanophostin (E) on Ca²⁺ release via each of the three IP₃R subtypes, and the same analogues compared with AdA (B, D and F). Results are means ± S.E.M. from 3 independent experiments. (G) A cation- π interaction between the adenine of AdA and R504 within the α -domain of the IBC is proposed to stabilize AdA binding (left). Closure of the clam-like IBC is proposed to be mediated by interactions between the 3'-phosphate of AdA and the α -domain of the IBC (blue ribbon), and between the 4'-phosphate and the β -domain of the IBC (green ribbon). In 3'-dephospho AdA, a cation- π interaction between AdA and the IBC α -domain is proposed to be sufficient to allow some effective closure of the clam. R504 is conserved in all three mammalian IP₃R subtypes (right).
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moiety decreases affinity for cerebellar IP₃R, which are largely IP₃R1 [37].

These results are consistent with our earlier conclusion that the 10-fold greater affinity of AdA relative to IP₃ requires the adenine moiety of AdA positioned to allow it to form a cation- π interaction with Arg-504 in the α -domain of the IBC of IP₃R1, a residue that is conserved in all IP₃R subtypes [8,12] (Figure 3G). We suggest that this interaction of AdA with IP₃R is likely to be similar for all IP₃R subtypes.

Hydroxyl Moieties that are Important for IP₃ Binding are Less Important for Binding of AdA

The 5'-CH₂OH and 2'-OH substituents of the glucose ring of AdA are thought to mimic the 3-OH and 6-OH of IP₃, respectively (Figure 1A). A structure equivalent to the 6-OH of IP₃ is an essential feature of all inositol phosphate analogues that bind to IP₃R [13,38,39] and inversion of its orientation from equatorial to axial reduces affinity by more than 100-fold at all IP₃R subtypes [40]. It is therefore surprising, but consistent with previous analyses of native hepatic IP₃R [36], that *manno*-AdA, which differs from AdA only in the orientation of its 2'-OH, should be only 5- to 10-fold less potent than AdA at each IP₃R subtype (Figures 4A and B, Tables 1 and 2). Why, when the 6-OH of IP₃ and 2'-OH of AdA seem to be analogous in the ligand structures, should these moieties make such different contributions to the interactions of IP₃ and AdA with IP₃R?

The 6-OH of IP₃ interacts, through a water molecule, with a lysine residue (K569) in the IBC [41] and, by interacting with the adjacent 1-phosphate, it has also been proposed to influence the behaviour of the 4,5-bisphosphate moiety of IP₃ [42]. The latter interaction is unlikely to contribute to AdA binding because the structures equivalent to the 6-OH (2'-OH of AdA) and the 1-phosphate of IP₃ (2'-phosphate of AdA) are in different rings in AdA (Figure 1A). We suggest that the lesser importance in AdA of a structure equivalent to the essential 6-OH of IP₃ comes from this hydroxyl mediating a relatively minor interaction with K569 in AdA, whereas for IP₃ it contributes also to appropriately orienting the critical 4,5-bisphosphate moiety.

The 3-OH group, although less important than the 6-OH, is another feature of IP₃ that contributes to high-affinity binding [43]. Our recent analyses of the functional effects of 3-deoxy-IP₃ established that it was ~40-fold less potent than IP₃ at all three IP₃R subtypes [7]. This is consistent with earlier work showing that 3-deoxy-IP₃ and analogues with other modifications of the 3-position have reduced affinity for the three IP₃R subtypes [40]. However, the equivalent modification of AdA, removal of its 5'-CH₂OH to give *xyl*-AdA (Figure 1A), had no significant effect on its potency at any IP₃R subtype (Figures 4C and D, Tables 1 and 2). This is consistent with a previous functional analysis of hepatic IP₃R, where *xyl*-AdA was only marginally less potent than AdA (Δ pEC₅₀ ~0.28) [36]. Our results suggest that despite the apparent structural similarity between the 3-OH of IP₃ and the 5'-CH₂OH of AdA (Figure 1A), the two hydroxyl groups do not contribute similarly to ligand binding. Previous analyses of IP₃ analogues suggested that replacing the 3-OH with the larger CH₂OH moiety caused the affinity to decrease by no more than 7-

fold [40]. A partial explanation for the lack of effect of removing the 5'-CH₂OH of AdA may therefore be that this moiety is less readily accommodated than a hydroxyl group in the IBC. This would suggest that an analogue of AdA in which the 5'-CH₂OH is replaced by 5'-OH might bind with increased affinity. We are unaware of such an analogue having been synthesized. The larger substituent at the 5'-position of AdA is, however, unlikely to provide the sole explanation for it making no discernible contribution to binding.

The 2'-phosphate of AdA is not a Super-optimal Mimic of the 1-phosphate of IP₃

It has been suggested that the 2'-phosphate of AdA interacts with the IBC in a manner that allows it to behave as a super-optimal mimic of the 1-phosphate of IP₃ [44,45]. However, our recent study combining structure-activity analyses with mutagenesis of the binding site suggest that the 1-phosphate of IP₃ is more important for binding than is the 2'-phosphate of AdA [12]. Removal of the 1-phosphate from IP₃ (to give (4,5)IP₂) caused its potency and affinity for IP₃R1 to decrease by ~100-fold [12], whereas removal of the 2'-phosphate from AdA (2'-dephospho AdA) causes a decrease in potency of ~17-fold in IP₃R1 (Figure 5) and ~40-fold decreases in potency were obtained with 2'-dephospho AdA and IP₃R2 and IP₃R3 (Figure 5, Table 1 and 2). These results establish that for all three IP₃R subtypes, the enhanced affinity of AdA is not due to its 2'-phosphate interacting more effectively than the 1-phosphate of IP₃ with the IBC.

A Bisphosphate Moiety is not Essential for Activation of IP₃ Receptors by AdA

All known active analogues of IP₃ have structures equivalent to its 4,5-bisphosphate moiety [13]. Structures of the IBC with and without IP₃ bound provide a rationale for this requirement by revealing that these two phosphate groups contact opposite sides (the α - and β -domains) of the clam-like IBC, closure of which initiates IP₃R activation [4,41]. Substantial evidence suggests that the 4',3'-bisphosphate moiety of AdA mimics the critical 4,5-bisphosphate of IP₃ [8] (Figure 1A).

4'-dephospho-AdA at concentrations up to 300 μ M failed to evoke Ca²⁺ release via any IP₃R subtype (Figure 6A). This is consistent with previous analyses by both functional and binding assays of IP₃R1 [28,46]. 3'-dephospho-AdA did, however, cause detectable Ca²⁺ release albeit with much reduced potency (Figure 6B). The synthetic route used to prepare 3'-dephospho-AdA makes it extremely unlikely that the activity could be due to minor contamination with AdA or related structures with a vicinal bisphosphate moiety. Maximal attainable concentrations of 3'-dephospho-AdA (300 μ M) failed to release the entire IP₃-sensitive Ca²⁺ store, but comparison of the concentrations required to achieve the same submaximal Ca²⁺ release suggests that 3'-dephospho-AdA is ~10,000-fold less potent than AdA at all three IP₃R subtypes. With such a massive reduction in potency the lesser sensitivity of DT40-IP₃R3 cells to AdA means that even the highest practicable concentration of 3'-dephospho-AdA (300 μ M) is close to the threshold for detecting Ca²⁺ release (Figure 6B).

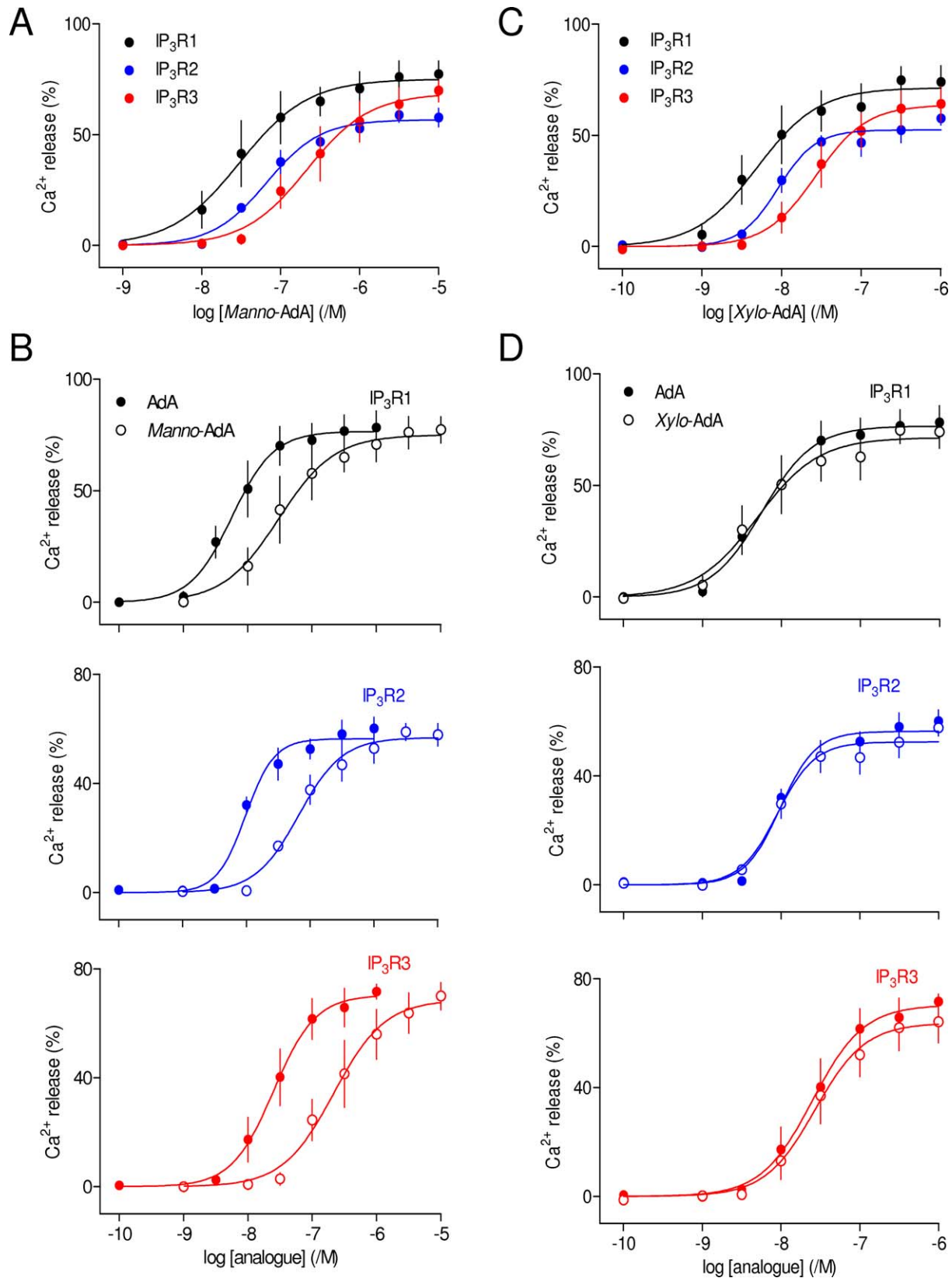


Figure 4. Hydroxyl groups within the glucose ring of AdA are unimportant. (A–D) Effects of *manno*-AdA (A) and *xylor*-AdA (C) on Ca^{2+} release via each IP₃R subtype, and the same analogues compared with AdA (B and D). Results are means \pm S.E.M. from 3 independent experiments. doi:10.1371/journal.pone.0058027.g004

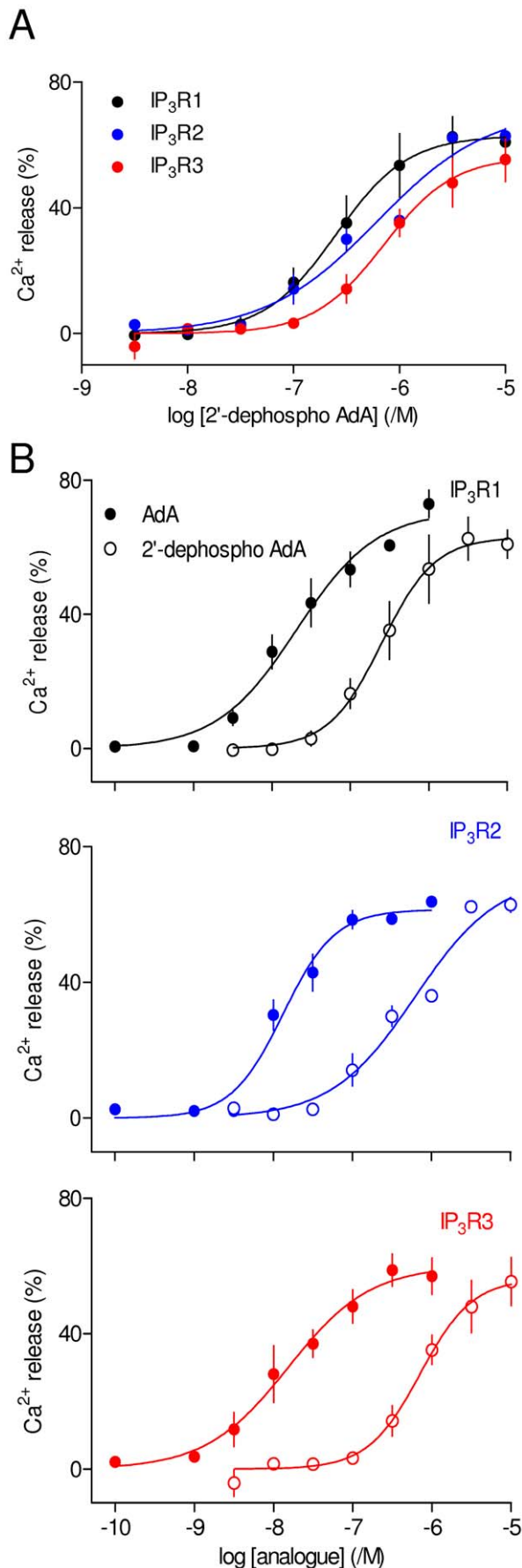


Figure 5. The 2'-phosphate of AdA is not the primary cause of its increased potency. (A) Effects of 2'-dephospho AdA on Ca²⁺ release via each IP₃R subtype. (B) The same analogue compared with AdA. Results are means \pm S.E.M. from 3–4 independent experiments. doi:10.1371/journal.pone.0058027.g005

The inability of high concentrations of 3''-dephospho-AdA to release the entire IP₃-sensitive Ca²⁺ store is likely to be due solely to its reduced affinity rather than reduced efficacy. A concentration of 3''-dephospho-AdA (30 μ M) that caused detectable Ca²⁺ release via IP₃R1 ($\sim 21 \pm 5\%$) had no effect on the sensitivity of the Ca²⁺ release evoked by a subsequent addition of IP₃. The pEC₅₀ was 7.00 ± 0.02 and 7.04 ± 0.06 ($n = 3$) for (1,4,5)IP₃ alone and in the presence of 3''-dephospho-AdA, respectively (Figure 6C). A partial agonist would be expected to shift the sensitivity to higher concentrations of IP₃. These results suggest that 3''-dephospho-AdA is a low-affinity full agonist of IP₃R.

These results extend our previous analyses of IP₃R1 by demonstrating that for all IP₃R subtypes, the 4''-phosphate group of AdA is essential for activity, whereas the 3''-phosphate is important but not essential. 3''-dephospho-AdA is the only known agonist of IP₃R to lack a structure equivalent to the 4,5-bisphosphate moiety of IP₃.

Discussion

AdA is a high-affinity full agonist of IP₃R that has been extensively used to explore the behaviour of IP₃R [reviewed in 8]. The activity of AdA has been confirmed in many cell types, but hitherto there has been no assessment of its activity in homogenous populations of IP₃R subtypes. We have demonstrated that AdA is ~ 10 -fold more potent than IP₃R at each IP₃R subtype (Figure 2, Tables 1 and 2), and the structural determinants of its high-affinity interaction with IP₃R are similar for all three IP₃R subtypes. Contrary to an earlier suggestion that the 2'-phosphate of AdA mediates its enhanced affinity by forming a stronger interaction with the IBC than the analogous 1-phosphate of IP₃, we find that the 1-phosphate makes a greater contribution to IP₃ binding than does the 2'-phosphate of AdA (Figure 5) [12]. A more likely explanation for the enhanced affinity of AdA is a cation- π interaction between its adenine moiety and R504 within the α -subunit of the IBC (Figure 3G) [28]. That explanation is supported by results for each IP₃R subtype showing that truncation of the adenosine moiety of AdA brings the potency of the resulting analogues (imidophostin, ribophostin and furanophostin) close to that of IP₃ (Figure 3).

A key step in the initial activation of IP₃R by IP₃ appears to be closure of its clam-like IBC as the 4-phosphate of IP₃ contacts one side of the clam (its β -domain) and the 5-phosphate contacts the other side (α -domain) [4]. That mechanism provides a satisfying explanation for the long-standing observation that all inositol phosphates that activate IP₃R share this essential 4,5-bisphosphate moiety. AdA is different in that its 4''-phosphate (analogous to the 4-phosphate of IP₃, Figure 1A) is essential, but 3''-dephospho-AdA retains activity at all three IP₃R subtypes, albeit with very low affinity (Figure 6). We suggest that for AdA, the need for the bisphosphate moiety to cause closure of the IBC can be partially replaced for all IP₃R subtypes by having an interaction between the adenine of AdA and the α -domain substitute for the interaction between the 3''-phosphate (analogous to the 5-phosphate of IP₃) and the α -domain [28]. Finally, whereas the 6-OH and, to a lesser extent, the 3-OH of IP₃ are important for IP₃ binding, the equivalent structures within AdA play lesser roles.

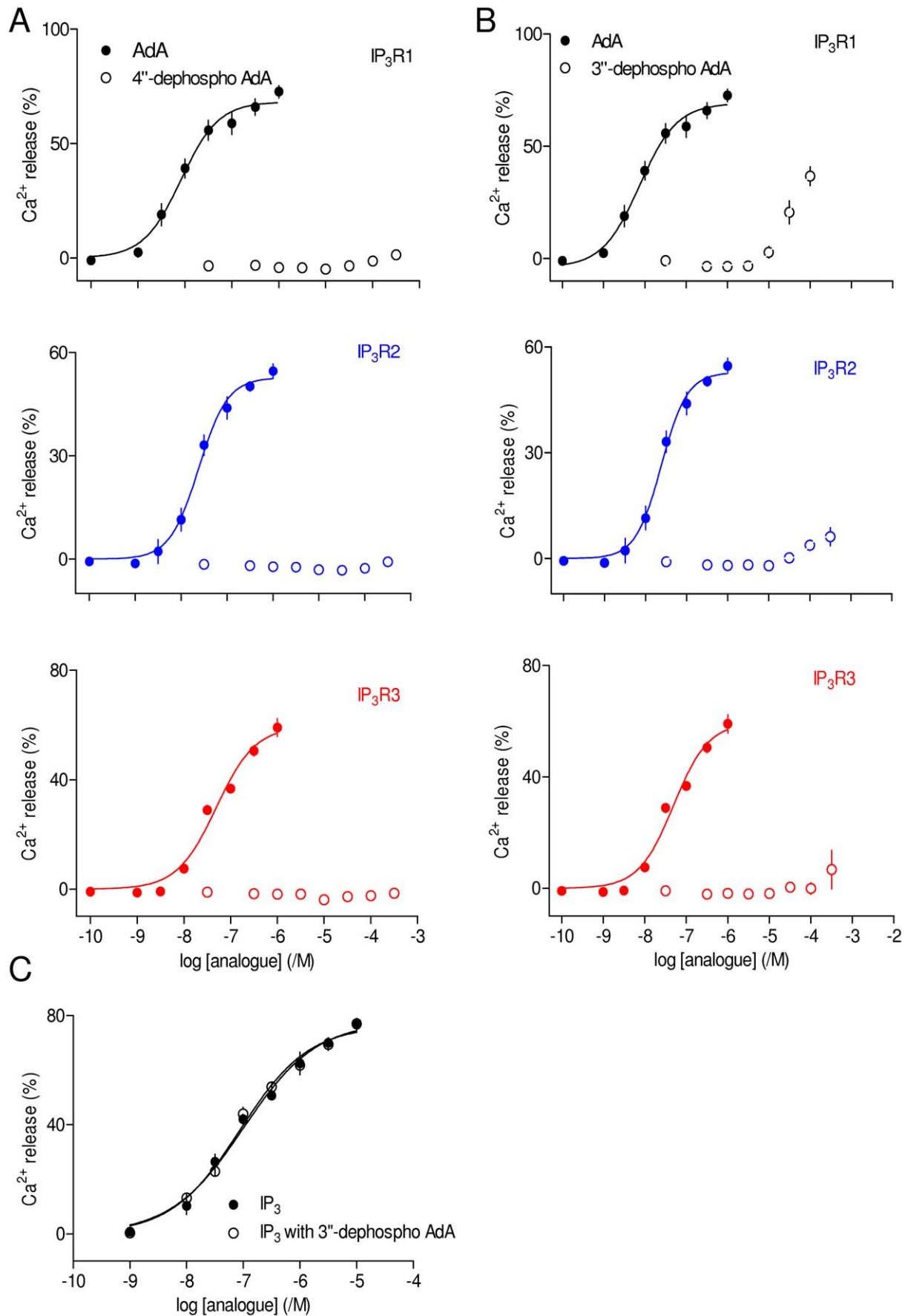


Figure 6. Structures equivalent to the 4,5-bisphosphate of IP₃ are not essential for AdA activity. (A, B) Concentration-dependent effects on Ca²⁺ release via each IP₃R subtype of 4"-dephospho AdA (A) and 3"-dephospho AdA (B) compared with AdA. Results are means \pm SEM from n independent experiments (n is provided in Table 1). (C) Concentration-dependent effects of IP₃ alone on Ca²⁺ release via IP₃R1 or after pre-incubation (30 s) with 3"-dephospho AdA (30 μ M), which itself evoked release of $21 \pm 5\%$ of the intracellular Ca²⁺ stores. Results (C) are means \pm SEM from 3 independent experiments.
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Both store-operated Ca²⁺ entry, which is triggered by depletion of IP₃-sensitive Ca²⁺ stores [47], and the spatial organization of subcellular Ca²⁺ signals have been reported to be differentially affected by IP₃, AdA or its analogues [14,16,17,19,21,22]. Our present results, which demonstrate that AdA structure-activity relationships are similar for all IP₃R subtypes, suggest that different physiological effects of IP₃, AdA or its analogues are more likely to result from differences in their affinities, kinetics or

rates of degradation than from selective interactions with different IP₃R subtypes.

Author Contributions

Conceived and designed the experiments: CWT SCT BVLV AMR. Performed the experiments: HS AMR. Analyzed the data: CWT HS SCT. Contributed reagents/materials/analysis tools: BVLV AMR. Wrote the paper: CWT.

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